Research Article

GPNMB/OA protein increases the invasiveness of human metastatic prostate cancer cell lines DU145 and PC3 through MMP-2 and MMP-9 activity ☆

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ARTICLE INFORMATION

ABSTRACT

Non-metastatic glycoprotein melanoma protein B (GPNMB), also known as osteoactivin (OA) is expressed in a wide array of tumors and represents an emerging target for drug development.

In this study, we investigated the role of GPNMB/OA in the progression of human metastatic DU145 and PC3 prostate cancer cells.

GPNMB/OA contribution in PCa malignant phenotype has been analyzed by small interfering RNA-induced GPNMB/OA silencing. We found that following GPNMB/OA silencing the migration capability of both DU145 and PC3 cells, evaluated by using *in vitro* invasivity assay, as well as the metalloproteinases MMP-2 and MMP-9 activity were equally strongly inhibited. By contrast knocking down GPNMB/OA weakly attenuated cell proliferation rate of DU145, an effect that paralleled with an increase number of apoptotic cells. However, PC3 cell growth seems to be not affected by GPNMB/OA. Together, these data reveal that GPNMB/OA acts as a critical molecular mediator promoting the acquisition of the more aggressive, pro-metastatic phenotype distinctive of human DU145 and PC3 cell lines.

Introduction

Prostate cancer (PCa) is the most frequently occurring tumor in men and the second leading cause of male cancer deaths both in Europe and in the United States [1]. Its incidence is increasing in the Western societies as population age; competing causes of death are more controlled and newer screening and detection tools are broadly implemented. At the beginning, the disease is androgen-dependent and can be treated with surgery and/or hormone therapy [2]. Unfortunately, benefits of the androgen-deprivation therapy are counteracted by the emergence of castration-resistant prostate cancer (CRPC), that is usually fatal. A remarkable aspect of CRPC is the reactivation of androgen receptor (AR) signaling, that is followed by an increase of AR-regulated genes such as the prostate specific antigen [3]. The role of the AR axis in CRPC is indeed increasingly recognized: castration-resistant tumors, even in the presence of very low levels of circulating androgens, are frequently still dependent on functional ARs. Several molecular mechanisms have been proposed to explain this phenomenon, such as an increased expression and/or mutations of the AR mRNA and protein, changes in the expression of AR coregulators, local increased expression of steroidogenic enzymes or ligand-independent AR signaling (reviewed in [4]). Thus, in CRPC, AR can assume a role of lineage oncogene [4,5].

Besides the AR axis, other growth factor pathways have been shown to be dysregulated in PCa development and progression [6,7]. Among these, we drew our attention on Nerve Growth Factor (NGF) [8]. Evidence indicates that the NGF pathway, which is crucial for the physiological prostate epithelial cell growth and differentiation, is involved in PCa carcinogenesis. The observation that NGF production is progressively lost in PCa advancement and that the decreasing expression of the NGF receptor p75[NGFR] in PCa cells correlates with cancer degeneration strongly supports this hypothesis [8]. On this line, we demonstrated that human PCa cell lines DU145 and PC3, when exposed to exogenous NGF, differentiate into a phenotype characterized by the re-expression of p75[NGFR], functionally active ARs and the reduction of telomerase activity [9,10]. This experimental model of NGF-induced reduction of PCa cell malignant phenotype was used to identify novel molecular targets involved in cancer progression. In particular, the gene-expression profiling technique applied on DU145 cell line allowed the identification of clusters of genes involved in proliferation, invasion and metastasis, specifically modified by NGF exposure [11]. Among genes involved in invasion and metastasis, our attention was drawn by the non-metastatic glycoprotein melanoma protein B (GPNMB) gene, encoding a type 1 transmembrane 572 amino acid protein [12]. GPNMB, also known as osteoactivin (OA), dendritic cell–heparin integrin ligand, or hematopoietic growth factor inducible neurokinin-type, is expressed in a wide array of normal tissue, such as bone, hematopoietic system and skin, where it influences cell proliferation, adhesion, differentiation and synthesis of extracellular matrix proteins [13]. Furthermore, GPNMB/OA overexpression has been linked to several aggressive cancers, including uveal and cutaneous melanoma [14,15], hepatocellular carcinoma [16], glioma [17,18] and breast cancer (BC) [19,20]. Indeed, the GPNMB/OA ectopic overexpression enhances the cell invasive phenotype both in vitro and in vivo [16,17,19,21]. In line with this, the role of GPNMB/OA in the human PCa, in particular in the advanced PCa, characterized by metastatic dissemination to liver, lung, brain and bone and currently incurable, has been recently investigated with conflicting results [22,23].

Against this background, we used the human metastatic PCa cell lines DU145 and PC3 to investigate the role of GPNMB/OA in PCa malignant progression. We clearly established that NGF treatment of PCa cells is associated with decreased levels of GPNMB/OA expression, supporting the role of GPNMB/OA in promoting the malignant phenotype. By using a siRNA approach, we found that the reduction of GPNMB/OA expression slightly influenced the rate of DU145 and PC3 cell proliferation but deeply affected their aggressive phenotype, abrogating their in vitro invasive potential. This phenomenon may find its molecular determinant in the reduced expression and function of the metalloproteinases MMP-2 and MMP-9, key molecules intimately involved in prostate tumor progression [24–27]. Our data indicate that GPNMB/OA can act as a promoter of the progression of human PCa into a more aggressive and metastatic phase.

Materials and methods

Cell culture

PC3 and DU145 cells were purchased from the American Type Culture Collection (ATCC) (LGC Promochem, Sesto San Giovanni, MI, Italy) and maintained in culture as suggested by ATCC. Media, supplements were purchased from Euroclone (Pero, Mi, Italy). Cells were treated as previously described [9,10]. Human recombinant NGF was purchased from Sigma-Aldrich Italia (Milano, Italy). Conditioned media were obtained by culturing DU145 and PC3 cells (10^6 cells/1 ml) in serum-free medium for 24 h. Media were then centrifuged at 1500g for 5 min at 4 °C, the debris was discarded and media were lyophilized and stored at -20 °C until use. PCS-440-010 human primary prostate epithelial cells were purchased from ATCC and cultured as suggested.

RNA extraction, reverse transcription and quantitative RT-PCR (Q-RT-PCR)

Total RNA was extracted from 10^7 cells using the RNAeasy kit (Qiagen, Milano, Italia) and 1 μg was transcribed into cDNA, using murine leukemia virus reverse transcriptase (Promega Italia, Milano, Italy). Gene expression was evaluated by Q-RT-PCR (iCycler iQ real-time PCR detection system, BioRad Laboratories, Milano, Italy), using the SYBR Green as fluorochrome, as described [11]. Sequences of sense and anti-sense oligonucleotide primers of GPNMB/OA were S: 5'-CTGTGAACACAGCCAATGTG-3'; AS: 5'-ATGGGGAGATCTTTGAGGAA-3' and of human β-actin were S: 5'-TCTTCCAGCCTTCCTTCCCTG-3'; AS: 5'-CAATGCCAGGGTACATG-GTG-3'. Reactions were performed under the following conditions: 1 cycle 95 °C for 10 min, 40 cycles at 95 °C for 15 s, and 60 °C for 1 min. Differences of the threshold cycle Ct values between the β-actin housekeeping gene and GPNMB/OA gene (ΔCt) were then calculated, as an indicator of difference in the amount of mRNA expressed.

Indirect immunofluorescence

Control and NGF-treated DU145 and PC3 cells were plated (10^5 cells/well) in tissue culture Petri dishes (3.5 cm diameter) containing

sterile poly-ʟ-lysine-treated coverslips. Twenty-four hours later, coverslips were fixed in 95% ethanol for 10 min and 50% ethanol for 5 min, then washed in phosphate buffer solution (PBS). Endogenous peroxidases were inactivated for 20 min with 0.03% H_2O_2 solution. Cells were permeabilized with a solution containing 0.2% Triton X-100 for 60 min at room temperature and then incubated overnight at 4 °C with a primary anti-GPNMB/OA monoclonal antibody (clone 303822, MAB25501, R&D Systems, Minneapolis, USA), raised in mouse and directed against the aa 22-486 sequence of the human protein (0.4 μg/ml). After extensive washes, the Alexa Fluor488 anti-mouse secondary antibody (Life Technologies, Milano, Italy) (5 μg/ml final concentration) was applied for 60 min at room temperature. Coverslips were mounted using the FluorPreserve™ Reagent and cell staining was detected using the IX51 inverted fluorescence microscope (Olympus, Tokyo, Japan) (magnification 200 ×).

GPNMB/OA gene silencing by RNA interference

A pool of four small interfering RNA (siRNA) duplexes targeting different regions of the human GPNMB/OA gene (si-GPNMB/OA) and non-targeting scrambled negative control siRNA (si-scrambled) duplex were used (ON-TARGETplus SMARTpool, Dharmacon). The si-GPNMB/OA sequences (according to the manufacturer's nomenclature) were siRNA-9: 5'-CCAGAAGAACGAUCGAAAU-3'; siRNA-10: 5'-UGCAAGAAGAGGCGGCGGGAUA-3'; siRNA-11: 5'-AUAUAACAUU-UGCGGUGAA-3'; siRNA-12: 5'-GGAAUUUCAUCUCUACGUCUU-3'. DU145 and PC3 PCa cells were transfected with different concentrations (10–100 nM) of si-GPNMB/OA or si-scrambled pool using the INTERFERin™ siRNA transfection reagent (Polyplus-Transfection, Euroclone). Briefly, cells (2×10^4) were seeded in each well of 12-well cell culture plates and incubated for 24 h. After 24 h, transfection complexes were prepared using si-GPNMB/OA, si-scrambled, siRNA transfection reagent and transfection medium, according to the manufacturer's instructions and delivered to cells.

Western blot

Untreated, si-scrambled- and si-GPNMB/OA-treated cells as well as the 24-h conditioned media for each treatment were collected. Both cell pellets and conditioned media were lysed in ice-cold buffer (20 mM Tris–HCl pH 7.4, 5 mM EDTA, 2 mM EGTA, 0.5% sodium deoxycholate, 0.1% SDS, 1% NP-40 and a complete set of protease inhibitors (Roche, Milano, Italy)). Equal amount of protein for each sample was separated by electrophoresis on a 4–12% NuPAGE Bis–Tris Gel System (Life Technologies, Milano, Italy) and electroblotted to a nitrocellulose membrane, following the manufacturer instructions. Membranes were reacted using different primary antibodies. The GPNMB/OA protein was detected using the anti-GPNMB/OA antibody described above (1 μg/ml final concentration). The anti-GPNMB/OA antibody from R&D System (AF2550) and the monoclonal anti-GPNMB/OA antibody (clone 1A8, Abnova) raised against human GPNMB/OA were also preliminary tested. Primary antibodies anti-MMP-2 and anti-MMP-9 both raised in mouse (1 μg/ml final dilution) were purchased from Millipore (Billerica, MA, USA). Lysates from untreated, si-scrambled- and si-GPNMB/OA-treated DU145 and PC3 cells were analyzed for the extracellular signal-regulated kinases 1 and 2 (Erk1/2) phosphorylation and total Erk1/2, both antibodies raised in mouse (0.4 μg/ml final dilution; Santa Cruz

Biotechnologies, Heidelberg, Germany). The secondary antibody raised in sheep (R&D Systems) (0.1 μg/ml final concentration) was applied for 1-h incubation at rt. A mouse polyclonal antibody directed against the N-terminal region of human α-tubulin (Sigma Aldrich Italia) was applied to cell extracts, to exclude that difference in the band intensity of the corresponding proteins could be due to errors in protein dosage or sample loading. The specific signal was visualized by the ECL-PLUS system (Amersham Italy, Milano, Italy). Densitometric analysis of the immunoblots was performed using the GelPro-Analyzer version 6.0 (MediaCybernetics, Bethesda, MD, USA).

In vitro invasion assay

The invasive potential of DU145 and PC3 cell lines was analyzed as previously described [9]. Briefly, untreated, si-scrambled and si-GPNMB/OA-treated cells (10^5 cells/well) were resuspended in DMEM–0.1% BSA medium and seeded in the inner side of transwells (BD Biocot Matrigel Invasion Chamber, BD, Bedford, MA, USA). After a 24-h incubation, cells in the inner chamber were removed with a cotton swab. Cells attached to the bottom side of the membrane were fixed with methanol, stained with toluidine blue, and counted.

Cell proliferation assay

Cell proliferation rate was evaluated as previously described [10,11]. Briefly, untreated, si-scrambled and si-GPNMB/OA-treated DU145 and PC3 cells (5×10^5 cells/well) were plated in culture dishes. Twenty-four hours later, cells were washed and detached with trypsin–EDTA. Cell suspensions containing 0.25% trypan blue were dropped in a hemocytometer chamber and the viable cells were blind counted under a phase contrast microscope by two different operators. Results are the mean±SE of six different dishes for each cell culture.

Double acridine orange/ethidium bromide (AO/EtBr) staining

A double staining with AO and EtBr was performed to visualize and quantify the number of viable, apoptotic and necrotic cells [28]. Briefly, untreated and si-scrambled- or si-GPNMB/OA-treated DU145 and PC3 cells (5×10^5 cells/well) were centrifuged for 5 min at 700g and resuspended in PBS. The dye mixture (100 μg/ml AO and 100 μg/ml EtBr) was added to cell suspension (1:10 final dilution) and immediately examined by a Zeiss LSM 510 META confocal laser-scanning microscope, with 10 × objective (Carl Zeiss AG, Germany). Several fields, randomly chosen, were digitalized and about 1000 nuclei for each sample were counted and scored.

Gelatin zymography

MMP-2 and MMP-9 activity was measured in the conditioned media of untreated and si-RNA treated DU145 and PC3 cells. Conditioned media were resuspended in PBS, and equal amounts of proteins (75 μg) were electrophoresed under non-reducing conditions into 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels polymerized with 0.1% gelatin. Gels were washed twice in 2.5% Triton X-100 for 30 min at rt to remove SDS

and incubated o/n at 37 °C in substrate buffer containing 50 mM Tris–HCl and 10 mM $CaCl_2$ at pH 8.0. Gels were stained with 0.5% Coomassie Brilliant Blue in 50% methanol and 10% glacial acetic acid for 30 min and destained with 50% methanol and 10% acetic acid. Translucent bands were quantified by densitometry using G: BoxChemiXT Imaging System (Syngene, UK).

Statistical analysis

Data analysis and graphics were obtained using GraphPad Prism 4 software. (GraphPad Software, La Jolla, CA). The statistical analysis was made using the one-way ANOVA, with a post-hoc test (Bonferroni's test) for multiple comparisons, considering $p < 0.05$ as threshold for significant difference. Data are expressed as mean$\pm$SE of at least three experiments run in triplicate, unless otherwise specified.

Results

Effect of NGF on GPNMB/OA expression in DU145 and PC3 cells

Microarray technology, employed in human metastatic DU145 cells to identify genes regulated by the differentiating treatment with NGF [9,10], led to the identification of cluster of genes involved in various aspect of cellular physiology and pathology, including invasion and metastasis [11]. Among these genes, a significant down-regulation of GPNMB/OA was observed (untreated DU145 value: 180; NGF-treated DU145: 33.4, -5.4 fold change, $p < 0.00002$) suggesting an important role of this glycoprotein in the mechanism underlying cancer progression. DU145 and PC3 cells were then treated with NGF and analyzed for GPNMB/OA mRNA and protein expression. Results obtained demonstrated that in DU145 cells, mRNA encoding GPNMB/OA, was significantly reduced by NGF of 7.2 fold (± 0.4) as well as in PC3 cells, where NGF treatment down regulated GPNMB/OA gene by 7.3 fold (± 0.9) compared to untreated cells. Commercially available human primary prostate epithelial cells were used as internal control, to evaluate the GPNMB/OA expression in normal tissue: Q-RT-PCR experiments with 5-fold serial dilutions of cDNA revealed a low detectable level of GPNMB/OA mRNA transcript only at the two highest cDNA concentrations (respectively 4 ng/μl: $\Delta Ct = 15.8 \pm 0.13$; 0.8 ng/μl: $\Delta Ct = 15.7 \pm 0.11$), while lower cDNA concentrations have not given rise to PCR products.

GPNMB/OA protein expression was evaluated by immunocyto-chemistry, using an anti-human GPNMB/OA antibody. As shown in Fig. 1, a positive immunoreactivity signal for GPNMB/OA was observed in both untreated cell lines, mostly localized at the membrane level and in the pericytoplasmic compartment (panels A and C), while NGF-treatment strongly reduced GPNMB/OA protein expression in both DU145 and PC3 cells (panels B and
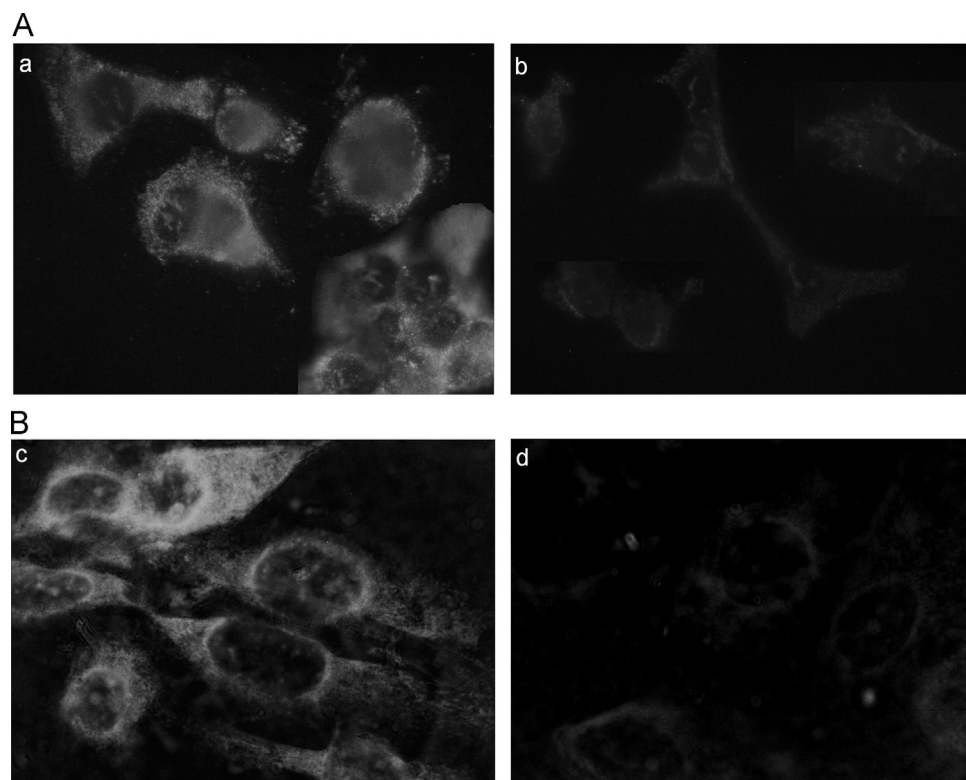

Fig. 1 – GPNMB/OA expression is reduced in DU145 (A) and PC3 (B) cell lines by NGF treatment. Untreated (a and c) and NGF-treated cells (b and d) were washed, fixed in 95% ethanol for 10 min and 50% ethanol for 5 min and incubated with a primary anti-GPNMB/OA monoclonal antibody directed against the aa 22-486 sequence of the human protein (0.4 μg/ml). After extensive washes, the Alexa Fluor488 anti-mouse secondary antibody was applied. Coverslips were mounted using the FluorPreserve™ Reagent and cell staining was detected using the IX51 inverted fluorescence microscope. No signal was detected when the primary antibody was omitted (not shown). Magnification: 400 ×. The picture is representative of at least three independent experiments, with superimposable results.

D). No signal was observed when the primary antibody was omitted (not shown).

GPNMB silencing in DU145 and PC3 cells

To gain insight into the role of GPNMB/OA in PCa cell lines, the siRNA approach was used. Preliminary experiments were conducted using a pool of four siRNA duplexes targeting different regions of the human GPNMB/OA gene (si-GPNMB/OA) and of non-targeting scrambled control siRNA duplex (si-scrambled), tested at different concentrations (10–100 nM) for different times (72–120 h), to find out the most selective and efficient treatment in silencing GPNMB/OA in both cell lines (data not shown). Transfection with siRNA for 120 h, at final concentrations of 50 nM and 10 nM, efficiently down-regulated GPNMB/OA protein

in DU145 and PC3 cells, respectively. Therefore these conditions were used for subsequent experiments.

In particular, transfection of DU145 cells with si-GPNMB/OA (50 nM) for 5 days, significantly reduced GPNMB/OA mRNA levels, measured by Q-RT-PCR (-89%; $p < 0.01$, Table 1). The specificity of this effect was validated by transfecting cells with the si-scrambled (50 nM; 5 days), that was unable to affect GPNMB/OA mRNA expression ($+12\%$). GPNMB/OA expression was also detected by western blot in DU145 cell lysates, multiple bands migrating between 60 and 120 kDa were detected (Fig. 2A), representing the different glycosylation forms of the protein [29,30]. Different primary antibodies raised against human GPNMB/OA were also tested, recognizing a similar pattern of bands, thus suggesting that several isoforms of GPNMB/OA are expressed in DU145 cells (data not shown). As shown in Fig. 2A, while no differences were detected between control and si-scrambled-treated cells, the expression of

Table 1 – GPNMB/OA siRNA treatment strongly reduced the level of GPNMB/OA mRNA in DU145 and PC3 cell lines.

	ΔCt	ΔΔCt	% vs. untreated cells
DU145 CELLS			
Untreated	11.7 ± 0.1	–	–
si-Scrambled-treated	11.5 ± 0.1	-0.2	$+12$
si-GPNMB/OA-treated	14.9 ± 0.1	$+3.2$	-89*
PC3 CELLS			
Untreated	17.0 ± 0.1	–	–
si-Scrambled-treated	17.1 ± 0.1	$+0.1$	-10
si-GPNMB/OA-treated	19.8 ± 0.5	$+2.8$	-86**

Cells were exposed for 120 h to si-GPNMB/OA or to si-scrambled, using the ON-TARGETplus SMARTpool system, as described in Materials and methods. One microgram of the total RNA extracted was retrotranscribed and used for the Q-RT-PCR. **Data are expressed as mean $\pm$ SE of at least three experiments run in triplicate.**
* $p < 0.01$ vs. untreated DU145 cells.
** $p < 0.05$ vs. untreated PC3 cells.

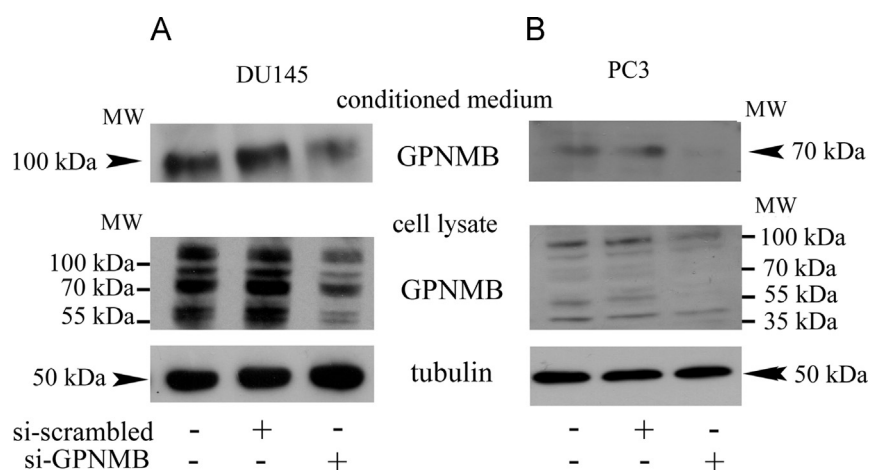
Fig. 2 – Analysis of protein expression in GPNMB/OA silenced DU45 (A) and PC3 (B) cellular extracts and conditioned media. Untreated, si-scrambled-treated and si-GPNMB/OA-treated cells and conditioned media were processed as described. The membranes were reacted with goat polyclonal affinity-purified antibody raised against the aa 23-486 of the human GPNMB/OA protein. The human α-tubulin was used as internal control. The specific signal was visualized by the ECL-PLUS system. Multiple bands migrating as a doublet between 60 and 120 kDa were detected, representing the different glycosylation forms of the protein [39,40]. The GPNMB/OA extracellular fragment, produced by plasma membrane cleavage and accumulated in the cell culture media was detected in the conditioned medium of untreated and siRNA-treated cells, as described. The blot is representative of at least three independent experiment, with superimposable results.

Table 2 – *In vitro* effects of GPNMB/OA silencing on DU145 cell line.		
Cell proliferation	**Cells/field (10^3)**	**% vs. untreated**
Untreated	268.3 ± 30.7	–
si-Scrambled-treated	250.6 ± 20.5	-6.6
si-GPNMB/OA-treated	181.2 ± 23.8	$-32.5*$
***In vitro* invasiveness**	**Cells/field (10^3)**	**%**
Untreated	9.5 ± 0.6	–
si-Scrambled-treated	9.0 ± 0.8	-5.3
si-GPNMB/OA-treated	3.5 ± 0.5	$-63.1**$

DU145 cells were plated and treated with si-scrambled or si-GPNMB/OA using the ON-TARGETplus SMARTpool system, as described in Materials and methods.

Cell proliferation: untreated cells, si-scrambled- and si-GPNMB/OA-treated DU145 cells were then detached, washed in PBS and counted. For direct counting with the hemocytometer, five fields for each treatment condition were counted, and counts were made in at least three separate experiments. Data are the mean $\pm$ SE from three different experiments run in quadruplicate.

In vitro invasiveness: untreated cells, si-scrambled- and si-GPNMB/OA-treated DU145 cells were detached and plated on Matrigel-coated filters. After a 24-h incubation, cells attached to the lower face of the filter were fixed, stained, and counted. Results are the mean $\pm$ SE from three different experiments run in triplicate.

* $p < 0.01$ vs. untreated DU145 cells.

** $p < 0.001$ vs. untreated DU145 cells.

different bands was significantly lowered in si-GPNMB/OA-treated DU145 cells (% vs. untreated cells: ~ 60 kDa: $-72 \pm 4.8\%$; $p < 0.05$; ~ 75 kDa: $-75 \pm 5.3\%$; $p < 0.05$; ~ 100 kDa: $-77 \pm 1.9\%$; $p < 0.001$; ~ 120 kDa: $-86.4 \pm 1,1\%$; $p < 0.05$). Moreover, the GPNMB/OA extracellular fragment, produced by plasma membrane cleavage and accumulated in the cell culture media [31], was measured in the conditioned medium of untreated and siRNA-treated DU145 cells. Fig. 2A shows a band of approximately 100 kDa in the media of untreated cells that was unaffected by treatment with si-scrambled ($92.6 \pm 1.7\%$), while was significantly reduced in si-GPNBM/OA-treated (% vs. untreated cells: $-66.9 \pm 4.1\%$; $p < 0.01$).

PC3 transfection with 10 nM si-GPNMB/OA for 120 h specifically and significantly reduced both mRNA and protein expression, as shown in Table 1 and Fig. 2B. Evaluation of GPNMB/OA expression by western blot, revealed the presence in PC3 cell lysates of three major bands corresponding to about 100 kDa, 60 kDa and 46 kDa (Fig. 2B). As shown for DU145 cells, a similar pattern of GPNMB/OA isoforms were detected by using different anti-GPNMB/OA primary antibodies (data not shown). Decreased GPNMB/OA expression was specifically revealed in si-GPNMB/OA-treated PC3 cells (% vs. untreated cells: ~ 46 kDa: $-52.3 \pm 3.9\%$; $p < 0.001$; ~ 60 kDa: $-69.4 \pm 2.8\%$; $p < 0.05$; ~ 100 kDa: $-54 \pm 3.2\%$; $p < 0.05$) (Fig. 2B), while si-scrambled did not affect GPNMB/OA levels (Fig. 2B), indicating the specificity of protein silencing. The GPNMB/OA extracellular fragment, accumulated in the supernatant of control and siRNA-treated PC3 cells was also measured by western blot, as described above. The ~ 70 kDa band detected in the culture media of untreated cells (Fig. 2B) was significantly reduced in si-GPNBM/OA-treated cells (% vs. untreated cells: $18.5 \pm 1.4\%$; $p < 0.001$), but unchanged by si-scrambled treatment.

Role of GPNMB/OA in DU145 cells

si-GPNMB/OA-treated DU145 cells were then tested for functional activity. The effect of GPNMB/OA knockdown on cell proliferation was first examined. For cell counting, cells were detached and counted using a hemocytometer [10,11]. As shown in Table 2, silencing of GPNMB/OA resulted in a reduction of DU145 cell proliferation rate of about 32.5% compared to control and si-scrambled-treated cells. The double staining with AO/EtBr was performed on untreated and si-RNA-treated DU145 cells, revealing that silencing GPNMB/OA deeply increased the number of apoptotic cells ($+69 \pm 3.1\%$; $p < 0.001$). By contrast, apoptotic cells were almost entirely absent in GPNMB/OA expressing cells (Fig. 3). These data thus suggest that in DU145 cells, impairment of apoptosis could be the main mechanism involved in cancer cell growth regulation induced by GPNMB/OA.

By using the Matrigel invasion assay, the effect of inhibiting GPNMB/OA expression in DU145 cells was also tested on their *in vitro* invasive potential, a key event leading to metastasis formation. Table 2 reports that silencing of GPNMB/OA substantially reduced the migration capability through Matrigel of DU145 cells ($-63.1 \pm 1.6\%$; $p < 0.001$), in contrast to control and si-scrambled DU145 cells.

Role of GPNMB/OA in PC3 cells

To establish whether the role of GPNMB/OA was peculiar for DU145 cells or it could represent a biomarker of PCa progression, the effect of GPNMB/OA silencing on cell proliferation and invasion capability was studied in PC3 cells as well. Results reported in Table 3 indicated that GPNMB/OA-silenced PC3 cells inhibited their cell proliferation rate, although the extent did not reach the statistical significance (-16% vs. controls). Interestingly, the GPNMB/OA knockdown substantially reduced the migration capability through Matrigel of PC3 cells ($-61.4 \pm 4.11\%$; $p < 0.001$; Table 3) compared to untreated and si-scrambled-treated cells (4.6% of reduction).

GPNMB/OA induces up-regulation of metalloproteinase 2 and 9 (MMP-2 and MMP-9) in DU145 and PC3 cells

To investigate the molecular mechanisms underlying GPNMB/OA effects on PCa human cell lines invasiveness, DU145 and PC3 cells were analyzed for the expression and function of MMP-2 and
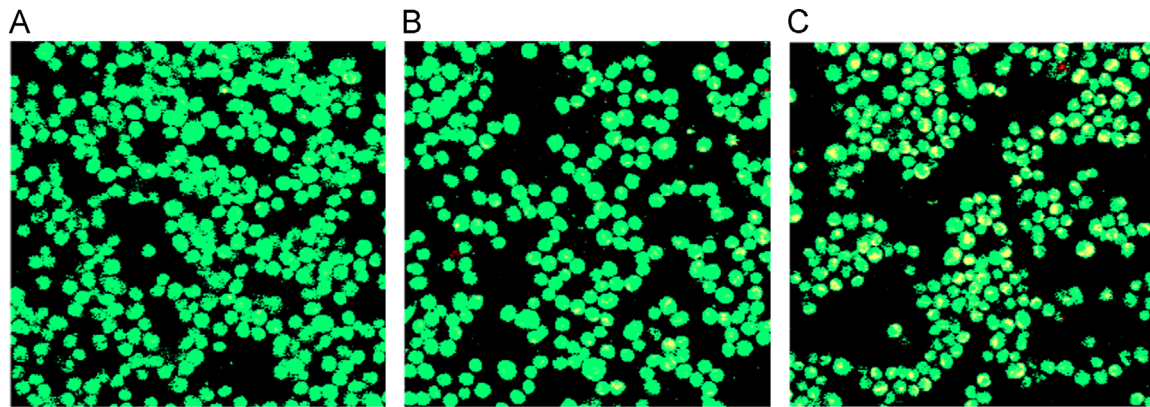
Fig. 3 – Effect of GPNMB/OA silencing on apoptotic cell death in DU145 cells. Untreated (A), si-scrambled (B) and si-GPNMB/OA-treated DU145 cells (C) were stained using AO/EtBr, as described in the Materials and methods. Viable cells (green), necrotic cells (red) and apoptotic cell (yellow) were visualized and quantified by using a confocal laser-scanning microscope. Magnification: 100×. The blot is representative of at least three independent experiment, with superimposable results.

Table 3 – *In vitro* effects of GPNMB/OA silencing on PC3 cell line.		
Cell proliferation	**Cells/field (10^3)**	**% vs. untreated**
Untreated	251.0 ± 75.5	–
si-Scrambled-treated	267.2 ± 86.0	$+6$
si-GPNMB/OA-treated	210.8 ± 86.3	-16
***In vitro* invasiveness**	**Cells/field (10^3)**	**%**
Untreated	35.5 ± 1.1	–
si-Scrambled-treated	34.0 ± 1.5	-4.6
si-GPNMB/OA-treated	13.9 ± 3.2	-61.4*

PC3 cells were plated and treated with si-scrambled or si-GPNMB/OA using the ON-TARGETplus SMARTpool system (Invitrogen), as described in Materials and methods.

Cell proliferation: untreated cells, si-scrambled- and si-GPNMB/OA-treated PC3 cells were then detached, washed in PBS and counted. For direct counting with the hemocytometer, five fields for each treatment condition were counted, and counts were made in at least three separate experiments. Data are the mean $\pm$ SE from three different experiments run in quadruplicate.

In vitro invasiveness: untreated cells, si-scrambled- and si-GPNMB/OA-treated PC3 cells were detached and plated on a Matrigel-coated filters. After a 24-h incubation, cells attached to the lower face of the filter were fixed, stained, and counted. Results are the mean $\pm$ SE from three different experiments run in triplicate.

* $p < 0.001$ vs. untreated PC3 cells.

MMP-9, critical molecules for cancer progression [32]. Results reported in Fig. 4 show that silencing GPNMB/OA protein deeply affected MMP-2 and MMP-9 expression in both cell lines, being reduced by $38 \pm 0.5\%$ ($p < 0.01$) and $59 \pm 1.1\%$ ($p < 0.001$), respectively, in DU145 cells and by $34 \pm 1.1\%$ ($p < 0.01$) and $56 \pm 0.7\%$ ($p < 0.01$), respectively, in PC3 cells, compared to the relative untreated cells. By contrast, in these cell lines, the expression levels of both the proteinases were unchanged following si-scrambled-treatment (Fig. 4). In addition, decreased levels of MMP-2 and MMP-9 were exclusively detected in the culture media of si-GNPMB/OA-treated DU145 cells ($-55 \pm 3.6\%$, $p < 0.01$, and $-71 \pm 1.05\%$, $p < 005$, respectively) as well as PC3 cells ($-72 \pm 0.7\%$, $p < 0.05$; $-64 \pm 2.7\%$; $p < 0.01$, respectively), compared to the levels detected in the media of both control and si-scrambled-treated cells, thus suggesting that GPNMB/OA specifically regulates the production and the release of these specific MMPs.

Gelatin zymography assay was then performed on the conditioned media, to evaluate MMP-2 and MMP-9 activity (Fig. 5).

A significant decrease of MMP-2 and MMP-9 functionality characterized the conditioned medium of both si-GPNMB/OA-treated DU145 cells ($-63 \pm 2\%$, $p < 0.05$ and $-49 \pm 2.3\%$, $p < 0.001$, respectively) and si-GPNMB/OA-treated PC3 cells ($-72 \pm 1.7\%$, $p < 0.05$; $-67 \pm 3.7\%$, $p < 0.001$, respectively). By contrast, MMPs activity was preserved in untreated and si-scrambled-treated DU145 and PC3 cell lines (Fig. 5).

The mRNA expression levels of tissue inhibitors of matrix metalloproteinases 1 and 2 (TIMP1 and TIMP2), and the endogenous molecules contrasting the activity of MMP-2 and MMP-9 [33] were also evaluated. We found that in both the cell lines, GPNMB/OA silencing did not affect TIMP 1 and TIMP 2 mRNA (data not shown) suggesting that GPNMB/OA-mediated regulation of invasiveness seems not to involve TIMPs regulation.

Finally, since evidence of MMPs regulation by the extracellular signal-regulated kinases 1 and 2 (Erk1/2) pathway has been described in different invasive tumors [34,35], phosphorylation of Erk1/2 was evaluated in the lysates from both untreated and si-scrambled- or si-GPNMB/OA-treated DU145 and PC3 cells.
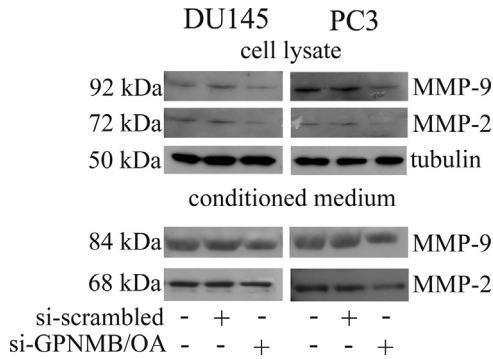
Fig. 4 – Effect of GPNMB/OA silencing on metalloproteinase MMP-2 and MMP-9 expression in DU145 and PC3 cells. Untreated, si-scrambled-treated and si-GPNMB/OA-treated DU145 and PC3 cells and conditioned media were processed as described. The membranes were reacted with goat polyclonal affinity-purified antibodies raised against human MMP-2 and MMP-9. In the cell lysates, MMP-2 and MMP-9 were detected as a bands of about 72 kDa and 92 kDa, respectively. The human α-tubulin was used as internal control. In the conditioned media, the active forms of MMP-2 and MMP-9 were detected as a bands of about 68 kDa and 84 kDa, respectively. The specific signal was visualized by the ECL-PLUS system. The blot is representative of three independent experiments, with superimposable results.
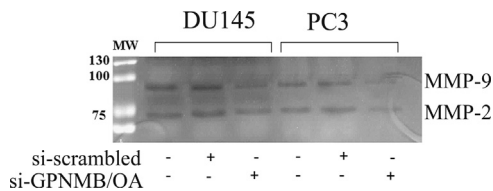

Fig. 5 – Effect of GPNMB/OA silencing on MMP-2 and MMP-9 activities. Conditioned media collected from untreated, si-scrambled-treated and si-GPNMB/OA-treated DU145 and PC3 cells (75 µg) were used for gelatin zymography assay. The gels were scanned and the images used for the densitometric analyses. The active forms of MMP-2 (≈ 68 kDa) and MMP-9 (≈ 84 kDa) were analyzed. Images were acquired and analyzed by using G: BoxChemiXT Imaging System (Syngene, UK). The experiment was repeated at least three times with superimposable results.

Our results indicated that high levels of Erk1/2 phosphorylation were detected in both control and si-scrambled DU145 and PC3 cells, that were not modify by GPNMB/OA silencing (data not shown).

Discussion

The glycoprotein GPNMB/OA, expressed in a wide array of normal tissue, is involved in various pathological conditions, including cancer. Association of GPNMB/OA expression and cancer, indeed, has been revealed in liver cancer, squamous cell lung carcinoma and soft tissue tumors [16,36,37] as well as cutaneous [15,38] and uveal melanoma [14]. Moreover, GPNMB has been found in many

BC cell lines and tumors [19–21] where a strong correlation between GPNMB/OA overexpression and aggressive phenotype has been clearly defined [19,20]. Accordingly, GPNMB/OA has been also detected in a significant portion of high-grade glioma tumors, but not in normal tissue, and its expression is a useful and powerful prognostic marker of poor survival [18].

Here, we demonstrated that GPNMB/OA was expressed in DU145 and PC3 cell lines, derived from human metastatic PCa. GPNMB/OA expression correlated with their malignant phenotype: both gene expression and immunistochemical analyses showed that GPNMB/OA expression was significantly decreased by NGF treatment, an experimental model of PCa cell lines differentiation previously characterized in our laboratories [9–11] thus suggesting that GPNMB/OA may be involved in the molecular mechanisms promoting the progression of PCa. The intracellular events underlying NGF-induced modulation of GPNBM/OA expression is still not yet clarified. For several cancer cell lines, the dysregulation of the NGF and NGF receptor pathway has been associated with the progression toward a more maligned phenotype [9,39–41]. In particular, the key point seems to be linked to the p75[NGFR] expression, as a strong correlation between cancer progression and decreased levels of p75[NGFR] has been observed [10,40,42,43].

On this line, in the prostate p75[NGFR], progressively lost during carcinogenesis [44,45] has been proposed as a tumor suppressor and anti-metastatic gene [45–47], involved in cell differentiation [48], cell growth inhibition and apoptosis [49,50]. p75[NGFR]-mediated signals involve the activation of the nuclear factor-kB (NF-κB) [50–52], a transcriptional factor able to either stimulate or inhibit gene synthesis depending on several mechanisms, such as the subunit composition of NF-κB complexes [53–55]. In line with these observations, analyses of the human GPNMB/OA promoter sequence ("Promo" Software, version 8.3 of TRANSFAC) [56,57] showed the existence of at least one putative NF-κB-responsive element, indicating that GPNMB/OA might be a NGF-mediated NF-κB-regulated gene. However, whether these molecular events are operating in DU145 and PC3 cells is now under investigation.

To clarify the role of GPNMB/OA in the PCa, we chose the mRNA silencing approach (siRNA) to investigate the role of GPNMB/OA in DU145 and PC3 cell lines. The siRNA approach led us to observe that, both the transmembrane protein as well as the extracellular peptide were strongly reduced. The transmembrane and secreted GPNBM/OA were detected in different molecular masses in DU145 and PC3 cells; the observed discrepancies about the GPNMB/OA molecular weight between the two cell lines, and more generally among the several cell types expressing GPNMB/OA [58], could be due to the different patterns of protein glycosylation processes [59]; GPNMB/OA, indeed, is heavily glycosylated by N-linked and O-linked glycans [29], having potential 11 N-linked [30] and 19 O-linked glycosylation sites [29], conferring to the protein peculiar properties in processing, folding and trafficking. In line with these observations, in DU145 cells numerous cell-associated GPNMB/OA isoforms were detected, ranging from 60 to 100 kDa, that were specifically silenced by siRNA treatment. Moreover, DU145 cells released a unique highly glysosylated/mature isoform form of 100 kDa [29]. By contrast, PC3 cells expressed several GPNMB/OA isoforms, highly reduced by siRNA silencing, detected both in cell lysates, with molecular masses from 50 to 100 kDa, and in cell supernatants, as an individual band of 70 kDa, suggesting an

apparently alteration in the glycosylation processes. Together with the observation that changes in the expression and activity of sialtranferases, responsible for the addition of sialic acid to glycans, have been associated with malignant potential in several tumors [60,61], our data may suggest that defective activities of these enzymes may occur in PC3 cells. Further experiments will elucidate the biological activity of both the released GPNMB/OA species detected in the conditioned media of DU145 and PC3 cells. Silencing GPNMB/OA robustly affected the ability of DU145 and PC3 cells to transmigrate through a synthetic membrane, establishing a correlation between GPNMB/OA expression and the tendency of invasiveness to adjacent normal tissue and of migration to distant organs. Among the multiple steps of PCa progression, the ability to break down the extracellular matrix represents a critical event for tumor invasion and metastasis that activates proteinases such as the matrix metalloproteinase (MMPs) [32], as well as their specific modulators, the tissue inhibitors of MMPs (TIMPs) [33]. Several evidences suggesting the crucial role of MMPs for PCa metastasis mechanisms have been collected: overproduction of MMP-2 and MMP-9 has been associated with more aggressive PCa phenotypes [25,62], high plasma and urine levels of MMPs have been measured in patient with PCa [62,63]; loss of TIMPs activity [64] or imbalance between MMPs and TIMPs, has been preferentially detected in primary PCa tissue, but not in normal prostate epithelium [65,66]. In line with these observations, altered production of MMP-2/-9 and/or MMPs/TIMPs imbalance has been described in both DU145 and PC3 cells [67,68]. Previous works have shown that in a non-invasive genetically defined human glioma cell line, the ectopic expression of GPNMB/OA significantly increased invasion capability mainly by increasing MMPs production [17] and the sustained MMP-3 expression induced by GPNMB/OA enhanced in vivo invasiveness of a bone metastatic BC cells [19]. On this line, we found that GPNMB/OA silencing induced a significant down-regulation of MMP-2 and MMP-9 expression level and activity, detected in both DU145 and PC3 lysates and conditioned medium. By contrast, mRNA levels of TIMP-1 and TIMP-2 were not affected by this glycoprotein suggesting that the unique regulation of both the MMPs by GPNMB/OA may be crucial components contributing to the acquisition of the highly invasive phenotype that characterizes both DU145 and PC3 metastatic cell lines. In line with our results, pharmacological or molecular strategies aimed to inhibit MMPs activities in both DU145 and PC3 cells, including drugs or mRNA silencing techniques, have been found to strongly affect PCa cells invasiveness and migration [24,69,70]. Among the numerous MMPs, enhanced MMP-2 and MMP-9 activity has been found to contribute to tumor invasion and metastasis in human PCa patients [25,62,63]. However, other MMPs, such as MMP-1, MMP-11 or MMP-13 have been shown to have an impact on diagnosis and survival of PCa patients [62,71–73] suggesting an extreme variability in the abnormalities of MMPs system likely caused by the biological heterogeneity of PCa tumors. By contrast, TIMPs expression and correlation with tumor progression appear to be more intricate since these multifunctional proteins exhibit both tumor suppressor and tumor promoting effects [74,75]. The precise mechanism of GPNMB/OA modulation of MMP-2 and MMP-9 synthesis and release is not well understood. However, in NIH-3T3 fibroblast cells it has been demonstrated that a recombinant extracellular fragment of GPNMB/OA had the ability to induce up-regulation of another member of the MMP family,

namely MMP-3, via Erk1/2 pathway activation [31]. In these experiments, MMP-3 modulation by the extracellular domain GPNMB/OA likely occurred through the interaction with a heparine sulfate proteoglycan-type receptor for osteoactivin, not identified yet [31]. In the present study, silencing GPNMB/OA did not alter the amount of Erk1/2 phosphorylation in both cell lines suggesting that other intracellular events may be involved in GPNMB/OA-induced activation of MMPs. Further experiments will elucidate whether these mechanisms really occur in PCa cell lines.

GPNMB/OA affected also cell proliferation, although with a lesser extent compared to invasiveness, thus suggesting its involvement in this phenomenon. In DU145 cells, indeed, GPNMB/OA knock-down induced a slight reduction in the proliferation rate, that was mainly due to an increase of apoptotic cell death. Since balance between cell proliferation and cell death or apoptosis deeply affects tumor growth, our findings indicate that DU145 cell proliferation induced by GPNMB/OA may be mainly related to its anti-apoptotic properties [21,76]. Although several *in vitro* studies carried out on genetically defined glioma cells clearly excluded any impact of GPNMB/OA on cell proliferation or apoptosis [17], recently in vivo studies revealed that GPNMB/OA overexpression in BC cells enhances primary tumor growth both indirectly, by up-regulating Vascular Endothelial Growth Factor (VEGF), and by the direct recruitment of endothelial cells, thus promoting angiogenesis: this effect was specifically induced by the secreted form of GPNMB/OA [21]. In line with our data, immunohistochemical analysis of GPNMB/OA-expressing tumors revealed, in addition to increased vascular density, a decreased number of apoptotic cells, suggesting a specific and more direct role of this glycoprotein in the control of apoptotic mechanisms [21,76]. Our results indicate that silencing GPNMB/OA in PC3 cells, however, had a slight impact on the cell proliferation rate. This result may find its rational in different metastatic origins of DU145 and PC3 cell lines (brain and bone, respectively), thus is reasonable that distinctive molecular modifications may have occurred, that strongly influence GPNMB/OA biological activities. Recent data from Tsui et al. [23], however, suggested an opposite role of GPNMB/OA in PCa cells: while this glycoprotein was detected in LNCaP cell lines, they found extremely low levels in DU145 and PC3 cell lines. This discrepancy may have a technical explanation, due to different cell culture conditions and/or experimental approaches. Moreover, they found that overexpressing GPNMB/OA in PC3 cell lines had the ability to reduce proliferation rate and tumorigenesis, thus exerting an anti-tumorigenic activity: this discrepancy can find its rationale in the distinct study design, as the transfection-induced expression of GPNMB/OA may allow protein overexpression, an usual consequence of this experimental technique, and may explain the conflicting results.

In conclusion, GPNMB/OA is emerging as an important protein that plays a role in the progression of a number of human tumors, including PCa. Several GPNMB/OA-dependent mechanisms, including the modulation of MMPs activity here described, the promotion of tumor growth and angiogenesis [21], as well as the capability to inhibit the tumor-reactive T-cell, leading to tumor escape from immunological destruction [77], might contribute to prostate cancer tumor progression. Although studies on human prostate and PCa tissues are needed, this may become a new biomarker, which could help clinicians to take the most appropriate and/or more aggressive treatment decisions, in the light

of the tendency of the cells expressing GPNMB/OA to invade adjacent tissue and to promote metastasis. Furthermore, GPNMB/OA may represent an ideal and therefore promising target for an antibody-based therapy, due, in particular, to the prevalent cell surface expression on cancer cells, in contrast to the predominantly intracellular expression in normal cells [18]. On this line, engineered GPNMB/OA-specific high-affinity single chain antibody fragment, known as CR0011-vcMMAE or glembatumumab vedotin, has been linked to the auristatin E cytotoxin, showing a potent anti-tumor activity both in preclinical studies [15,78,79], and in phases I/II clinical trials for patients with advanced melanoma and BC [14,80,81].

Conflicts of interest

AB received lecture fees from Janssen, Astellas Pharma and Xanofi, PFS was a consultant for Roche, DZ was a consultant from Ipsen and received lecture fees from GlaxoSmithKline, and SS received lecture fees from AstraZeneca and GlaxoSmithKine. These collaborations are not related to the topic of the present manuscript. CF, SB, FB, MF, SAB, CS, CM, and MM have nothing to disclose.

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